

The Effect of Hybridization on Beak Morphology in the House Sparrow (*Passer domesticus*) and Italian Sparrow (*P. italiae*)

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Abstract

Interspecific hybridization has intrigued evolutionary biologists since Darwin's time. New genetic combinations can arise from hybridization, leading to offspring sometimes able to adapt to different niches than their parental species. I wanted to study beak morphology in Italian sparrow and house sparrow in a contact zone in the Alps, where they interbreed. This contact zone is situated in an ecotone, so I also investigated if climatic factors could affect beak size or beak shape. I used a geometric morphometrics approach, with landmarks from photos, to investigate if there are differences between the two species and their hybrids in this zone. The results revealed a pattern of intermediacy for the hybrids in beak size and in one dimension of beak shape. Another dimension of beak shape is transgressive in hybrids, that is outside the range of their parents. I show that there is a significant association between genetic admixture and beak size and also the first dimension of beak shape. Moreover, beak size variation is also significantly associated with environmental variation. The transgressive dimension of beak shape was also affected by environment, but not by genetic admixture. This may indicate that different factors and selection pressures affect beak morphology, including environment and genetic admixture. Cline analysis was used to infer selection on the beak. Steep clines were found for beak size and the intermediate dimension of beak shape; beak size having the steepest cline. Generation time estimates, assuming neutral diffusion, based on the widths of the cline analysis, were much lower than the likely age of the hybrid zone, indicating strong selection on beak size and shape in the hybrid zone. Selection coefficients indicate low fitness for intermediates. Overall, my results imply strong selection on beak size and shape. I discuss whether these traits may be involved in reproductive isolation between the Italian sparrow and house sparrow.

Table of Contents

Introduction	1
1 Material and method.....	6
1.1 Study Species.....	6
1.2 Study Site.....	7
1.3 Sampling.....	8
1.4 Geometric Morphometrics.....	8
1.4.1 <i>Digitizing Landmarks</i>	9
1.4.2 <i>Repeatability Test</i>	11
1.5 Morphological Analysis	12
1.6 Hybrid Index.....	13
1.7 GLM	14
1.8 Cline Analysis.....	15
1.8.1 <i>Strength of selection</i>	16
2 Results	18
2.1 Beak shape and size differences	18
2.2 Variation across the hybrid zone	23
2.3 Cline Analysis.....	23
3 Discussion	26
3.1 Differences in Beak Morphology Between House and Italian Sparrow	26
3.2 The Effects of Hybridization on Beak Morphology	27
3.3 The effects of environmental variation on beak morphology.....	29
3.4 A potential role of beak morphology in reproductive isolation.....	30
4 Conclusion.....	34
5 Glossary.....	35

6	References	36
7	Appendix	41
	Appendix 1. Consensus plot.....	41
	Appendix 2. Climatic factors along the hybrid zone.....	42
8	Tables.....	43
	Table 1. Sample data.....	43
	Table 2. Repeatability test, Cronbach's alpha.....	44
	Table 3. Repatability test, correlation.....	44
	Table 4. CVA, variation among groups.....	45
	Table 5. Mahalanobis distances among groups.....	45
	Table 6. Pair wise differences between the groups.....	45
	Table 7. Classification table for house sparrow and hybrids.....	46
	Table 8. Classification table for house sparrow and Italian sparrow.....	46
	Table 9. Classification table for hybrids and Italian sparrow.....	46
	Table 10. MANCOVA and Pillai's Trace	47
	Table 11 Multivariate test for beak shape and size variables.....	48
	Table 12. ANCOVA for beak shape (CV1).....	49
	Table 13. ANCOVA for beak shape (CV2).....	49
	Table 14. ANCOVA for beak size.....	50
	Table 15 Cline widths and centres.....	51
	Table 16. Selection coefficient & generation time estimate for beak shape and beak size.....	52

1 Introduction

The role of hybridization in evolution is a widely debated topic in biology with many contrasting views. Is hybridization simply an evolutionary misstep, or does it have the potential to generate diversity and new evolutionary trajectories? Gene flow can constrain adaptive divergence (Grant & Grant, 1994), and hybridization may lead to speciation reversals (Kleindorfer et al., 2014) and hence limit biodiversity. On the other hand, hybridization involves recombination of two different genomes, which will increase genetic variation and which may enhance evolvability (Abbott et al., 2013). This can be evolutionarily important, because the enhanced variation can lead to the emergence of novel gene combinations and phenotypes that may be advantageous in terms of fitness (Grant & Grant, 1992; Grant & Grant, 1994). Moreover, in some cases hybridization may lead to the creation of a third distinct lineage that develop reproductive barriers to its parent species (Mallet, 2007; Mavárez & Linares, 2008). When such hybrid speciation occurs with no change in chromosome number it is called homoploid hybrid speciation (HHS). This mode of speciation appears to be more common in nature than previously thought (Mallet, 2007).

It is often the case that hybrids have lower fitness than their parents, with offspring suffering reduced fertility, viability or anomalies (Futuyma, 2009). Because the parental genomes have been exposed to selection over a long period of time, they should be better adapted to the environments than the hybrids, which typically will have novel gene combinations not previously tested by selection (Coyne & Orr, 2004). However, research has shown that in some cases hybrid taxa can become better adapted than their parents in novel environments (Rieseberg et al., 2003). Hybrids inherit alleles from both their parents, and through selection and recombination, disadvantageous combinations will disappear from the hybrid population

if it manages to overcome the fitness loss in early generations. This can result in genomic mosaicism in which different genes are inherited from either parent, as has been found in *Passer* sparrows (Hermansen et al., 2012; Trier et al., 2014), or in introgression of smaller ecologically important genomic regions such as in *Heliconius* butterflies (Jiggins et al., 2001).

Although hybrids are often intermediate between their parents in phenotype, another possible outcome of hybridization is transgressive segregation in which hybrids possess phenotypes outside the range of those of the parents. Transgressive segregation has been thoroughly studied, particularly in plants (e.g. Rieseberg et al., 1999). For example Rieseberg *et al.* (2003) studied hybrids from wild sunflowers and found numerous phenotypes that were outside the phenotypic range of either parent species. These traits enabled the hybrids to adapt to more extreme habitats than their parent species. Transgressive morphology can be the result of new genetic combinations with non-additive effect (Renaud et al., 2012).

Hybrid zones are contained geographic zones where genetically distinct populations breed and produce hybrids (Barton & Hewitt, 1985). They can be stable and persist for long evolutionary periods of time, although hybrid zones may be subject to complex and dynamic equilibriums (Moore, 1977). In a hybrid zone there is a central principle that a balance between selection and dispersal will form, which in turn defines how sharp the transition from one species to the other will be (Haldane, 1948). As dispersal increases the width of the hybrid zone will be wider. While with strong selection the width will be narrower. Such a gradient is also referred to as a cline (Barton & Hewitt, 1985). Clines occur when gene flow across the contact zone is limited. Clines can be maintained by extrinsic and intrinsic selection, favouring certain phenotypes or genes on either side of the contact zone. When gene flow is limited selection will produce clines in phenotype or genotype frequencies.

Hybrid zones are often found along an environmental transition, as shown in the study near Cracow (Poland) where *Bombina bombina* and *B. variegata* meet and interacts (Szymura & Barton, 1986), and also *Helioconius erato* and *H. imera* in central America (Jiggins et al., 1996). This is because environmental transition allows different taxa with different niches to meet and interact.

The beak is an important ecological trait in birds, and is adapted to their diverse diets, ranging from seeds, nectar, mussels, and insects to other vertebrates such as rodents. During adaptive radiations, organisms evolve various morphological differences to adapt to new habitats, and new species can arise rapidly (Price, 2008). A famous example of an adaptive radiation is Darwin's finches on the Galapagos Island (Grant et al., 1985; Grant & Grant, 2002), a study system which have provided much knowledge about hybridization and speciation. Beak shape is a fast evolving ecological trait (Grant & Grant, 1994), which is also linked to reproductive isolation (Huber et al., 2007). Furthermore, divergence in beak shape has also been found to be an adaptive response to different dietary niches (Kleindorfer et al., 2006). Its evolutionary direction is also unpredictable, as a long-time study of the Darwin's finches has shown (Grant & Grant, 2002). Fluctuations in climate is one factor that can have great impact on beak size in just a couple of generations (Hendry, 2009). Moreover, beak shape and size differences has been found to be controlled by a few genetic elements of large effect in embryonic development (Abzhanov et al., 2004; Mallarino et al., 2011). However, beak morphology in many birds can vary in a more continuous way along an environmental gradient (Symonds & Tattersall, 2010), unlike the Darwin's finches where variation in beak is quite discrete.

In this study, I investigate beak morphology in the Italian sparrow (*Passer italiae*), the house sparrow (*P. domesticus*) and birds from their hybrid zone in the Italian and Swiss Alps.

Previous studies have shown that the Italian sparrow is the result of homoploid hybrid speciation, originating through past events of hybridization between the Spanish sparrow (*P. hispaniolensis*) and the house sparrow (Elgvin et al., 2011; Hermansen et al., 2011). Beak height and length in the Italian sparrow can vary on a more continuous scale as a response to environmental variation (Eroukhmanoff et al., 2013). This hybrid species also has two ecologically differentiated parents, the house sparrow being a human commensalist (Sætre et al., 2012) and the Spanish sparrow which is less associated with humans and which prefers more mesic habitat. Both parental species are mainly feeding on seeds from human cultivated crops, and seeds from annual herbs (Summers-Smith, 1988). However, the house sparrow is more opportunistic and thus has more alternative food resources, including household scraps and mussels, so it can almost be called an omnivore (Summers-Smith, 1988). Adaptation to human commensalism in the house sparrow is thought to have had a strong effect on its beak shape. Human commensal sparrows have a longer, pointed and more robust beak than non-commensal house sparrows, probably to cope with the large and hard seeds of cultivated cereals (Riyahi et al., 2013). The Italian sparrow is a human commensal like the house sparrow, but little is known about potential differences in beak morphology between these two species that have similar ecological niches, but which differ in genetic make-up, one being a hybrid species, and the other, one of its parents.

My first aim for this study was to investigate whether beak shape or size differs between the hybrid Italian sparrow and one of its parents, the house sparrow. Since these two species meet in a hybrid zone located along a sharp environmental transition (the Alpine mountain range), there was also a double opportunity to study the effects of both climate and introgression on beak shape in this system. By using information about both climatic variation and genetic introgression in sampled hybrid populations, I aimed at disentangling the effects of ecological

selection and hybridization on beak morphology in sparrows, and attempted to evaluate whether this trait could contribute to isolation between the two species.

2 Material and method

2.1 Study Species

The house sparrow has a broad geographic distribution ranging throughout the whole of Palearctic to the Indian subcontinent (Summers-Smith, 1988). The Spanish sparrow has a wide range around the Mediterranean Sea, throughout the Middle East to Central Asia. The Italian sparrow is distributed on the Italian peninsula, Crete, Corsica and some smaller Mediterranean islands. The house sparrow is known to be a human commensalist and it mainly exists in manmade landscape like farmland and towns (Summers-Smith, 1988; Sætre et al., 2012; Riyahi et al., 2013). Its food preference includes seeds from cultivated crops, seeds from annual herbs and also scraps from households. The house sparrow is an opportunist and can also include a great variety of other food sources in its diet. The Italian sparrow also prefers seeds from cultivated crops and is often found close to farms with livestock. Male hybrids between Italian and house sparrows have an intermediate phenotype, most conspicuously in crown colour consisting of variable amounts of Italian sparrow chestnut and house sparrow grey. Spanish sparrow has a larger black bib than the Italian and house sparrow and a chestnut-red crown and nape (Summers-Smith, 1988). As such the Italian sparrow is intermediate in its colours between the house sparrow and the Spanish sparrow.

2.2 Study Site

Sampling was carried out during spring and summer 2012 in the district of Ticino in Switzerland and Lombardy in northern Italy (Fig. 1). This Alpine region varies a lot in terms of altitude, with peaks up to 4800m and valleys with lowlands. Elevation is also lower in Lombardy than further north. Cultivated crops of cereals dominate the landscape in the south, whereas animal farming dominates in the alpine valleys. The mountains in the Alps form a geographic barrier with colder and wetter climate in the north compared to the dry and warm climate in the south. Phenotypic house sparrows live north of the alpine ridge and Italian sparrows to the south. In alpine valleys just south of the alpine ridge mixed and intermediate phenotypes are found indicating hybridization between the two species.

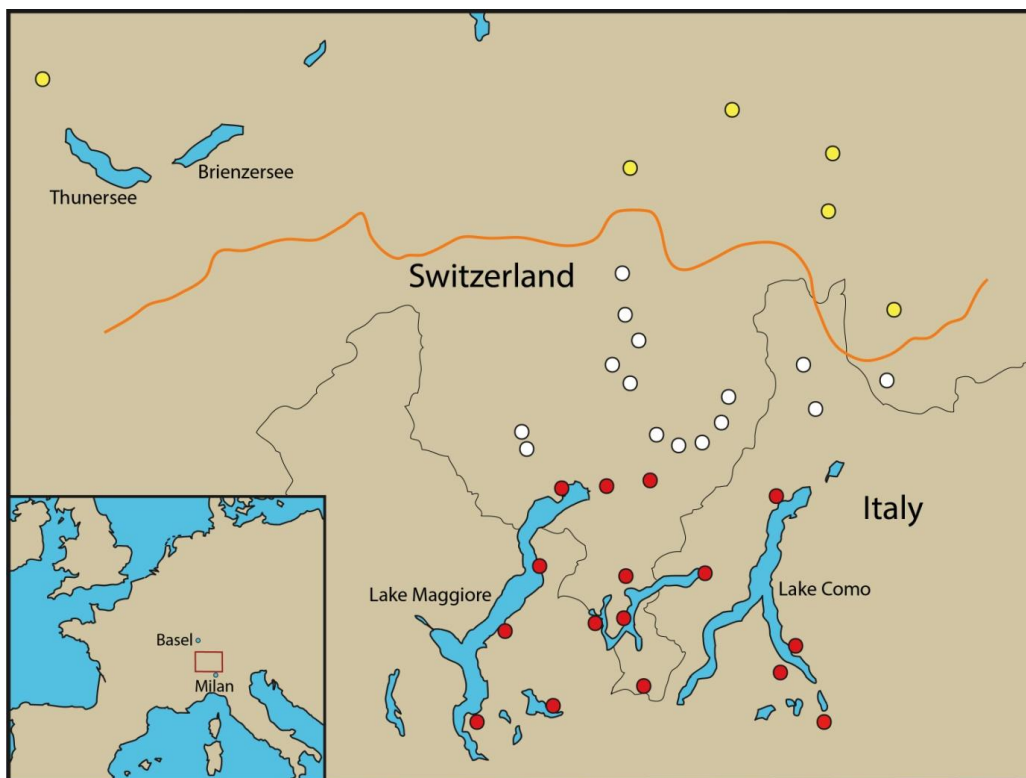


Figure 1. Map showing sampling locations in northern Italy and southern Switzerland. Red circles are Italian sparrow, white circles are hybrid populations and yellow circles are house sparrow populations. The orange line indicates the alpine ridge.

2.3 Sampling

Birds were caught using mist nets at 36 different localities in Ticino and Lombardy (Table 1). Blood samples were taken from a brachial vein under the wing for DNA analysis. Tarsus length was measured, and photos were taken of all individuals. The birds were placed on millimetre-paper, used as a scale for measuring size. Every individual were put in the same position with the head to capture the beak in the same angle on the photos. Blood samples were collected, and the birds were then released. Sample locations were classified into three categories; *house sparrow*, *Italian sparrow* and *hybrids* based on the phenotypes of male birds, and locations for where they were captured. North of the Alps is pure house sparrow, hybrids in the hybrid zone, and Italian for individuals captured south of the hybrid zone.

2.4 Geometric Morphometrics

In this study, I used a geometric morphometrics approach to get quantitative measures from images obtained in the field. This is a method which is extensively used in studies of beak shape (e.g. Abzhanov et al., 2004; Abzhanov et al., 2006; Marugán-Lobón & Buscalioni, 2009; Campàs et al., 2010; Riyahi et al., 2013). Geometric morphometrics is a tree-step procedure where the first step is data acquisition of coordinates as landmark points of the structure in 2D or 3D. In this study it included digitizing 2D landmarks from photos. Several different programs were used in the different steps from digitizing landmarks to analysis of the data. The second step is to transform that information into shape data and the third step is the statistical analysis of these data and interpretation in a biological context. Geometric

morphometrics captures information about shape and size of organisms or structures. This method has been widely used to study variation in shape representing different geographic localities, developmental stages, genetic or environmental backgrounds (Rohlf & Marcus, 1993; Campàs et al., 2010; Klingenberg & Marugán-Lobón, 2013; Klingenberg, 2013a). Geometric morphometrics has also proven to be a method with both high visual and analytical power for testing differences in shape (Adams et al., 2013; Klingenberg, 2013b). It is a computer-based tool which makes it comprehensible and also very good for visualization of shape variation (Rohlf & Marcus, 1993; Adams et al., 2013; Klingenberg, 2013b).

2.4.1 Digitizing Landmarks

When digitizing landmarks, there are five important criteria to have in mind when choosing photos and points for the landmarks to be used in the analysis. First, ideally the landmarks should be at homologous points on all the specimens. Second, these points should not alter their positions in relation to other landmarks. Third, the landmarks should give a good coverage of the structure we want to study. Also the repeatability for the landmarks on the specimens needs to be high. Finally, the landmarks must be in the same plane (Zelditch et al., 2004). A crucial part of this process is to locate the points precisely with great consistency so that the correspondence between specimens is clear. When using 2D landmarks from images, it is necessary to make some specific rules about how to select which photos to be included in the analysis (Zelditch et al., 2004). After testing different options, many individuals were discarded either because of lack of good resolution on homologous points or mis-orientation of the beak (so that the beak was not contained in the same standardized plane). Of the initial

235 individuals of which we obtained photos, only 138 had the quality needed for further analysis. As many homologous landmarks as possible were chosen as they were consistently found on all specimens. Six homologous landmarks and additionally two outlines (see glossary p. 36) with seven semi-landmarks were enough to capture full coverage of the shape of the beak. Homologous positions for the landmarks were digitized as shown in figure 2. Tip of the upper mandible were excluded as a homologous landmark due to great variation in wear-off between birds. With this black beak and a smooth contour line without any good homologous points, an outline with sliders (semi landmarks) were necessary to get good



Figure 2. Homologous positions for the six homologous landmarks here shown in turquoise dots, were; landmark 1; the base of the upper mandible, landmark 2; tip of lower mandible, landmark 3; corner of mandibular rami, landmark 4; the base of the mouth opening and landmark 5; point of maximum height in a bump at the lower mandible, and landmark 6; in the centre of the eye. The blue line is the outline, and the red dots are the semi landmarks used to cover most of the shape of the beak.

enough coverage of the shape. An outline was made on the upper and lower mandible with 7 sliders (semi-landmarks) on each.

TpsDig2 (Rohlf, 2010a) was used to digitize landmarks. Homologous landmarks were digitized first. Then an outline for the upper beak was made by using the draw curve function. The outline curve was resampled by adding seven points divided by length to get equal distances between them. These points are the semi-landmarks. The same procedure was followed for the lower mandible. The tps output file made here is the raw data with coordinates for each landmark for all the specimens. The sliders method was chosen with bending energy option D^2 . Additionally partial warps and relative warps were calculated by using tpsRelw (Rohlf, 2010b).

2.4.2 Repeatability Test

To check the reliability of the landmarks, I followed the same digitizing procedure on all the images a second time. Relative warps from the first dataset and the repeated measures were used to make a regression to check the correlation between these two datasets. Additionally a reliability analysis, Cronbach's alpha was made to assess the quality of the measurements.

The correlation between the data used in the analysis, and the repeated measures were very consistent across replicates. The Cronbach's Alpha coefficient was very high ($\alpha = 0.967$ (Table 2) for the repeatability test. The correlation between repeated measurements is highly

significant ($p < 0.001$) with small confidence intervals as shown in table 3. The landmarks can therefore be considered reliable in capturing relevant variation in beak morphology, and I proceeded with using the first measures.

2.5 Morphological Analysis

MorphoJ (Klingenberg, 2011) is a program designed for statistical analysis of geometric morphometric data acquired through tpsDig2 or other programs that creates data from landmarks. After testing some different programs like geomorph in R, tps-programs and IMP, I decided to continue with MorphoJ because you have the opportunity to do most of the analyses in a single program, and because it is flexible and comprehensible to use. First a consensus plot was made to see the distribution of the landmarks (Appendix 1). A GPA (general Procrustes analysis) was conducted on the three categories, house sparrow (PD), Italian sparrow (PI) and hybrids (H) to do further morphological analyses. A CVA (canonical variates analysis) was also performed in MorphoJ (Klingenberg, 2011) to assess differences between the three categories of birds. This analysis revealed that the first axis of variation summarized 64, 96% of the total variation. In order to check whether beak shape differs between the groups irrespective of allometric effects, further analysis included tarsus length as a covariate to control for differences in size between and within categories. When doing the CVA, a permutation test for pairwise differences were performed with 10.000 iterations. Mahalanobis distance is recommended to use when having a large sample size and Procrustes distances when having small sample size with just a few specimens. To obtain pairwise differences a discriminant function analysis (DFA) was conducted to check how well the

birds were classified into the correct morphological group. The cross validation in the DFA is an assessment of the reliability of the classification between the groups. The DFA gives Mahalanobis distances between the pairs in the comparison in addition to a classification /misclassification table showing the number of individuals which are classified into the correct group or not. For each locality, I extracted four environmental variables, namely mean annual temperature, temperature seasonality, mean annual precipitation and precipitation seasonality from the Worldclim database (Hijmans et al., 2005). I used a resolution of 30 arc-seconds (~1km). I also estimated the altitude for each sampling location by using Google Earth (Google Inc., 2013).

2.6 Hybrid Index

To study the effect of hybridization on beak morphology, a molecular hybrid index of each individual was included in the analysis. Hybrid index is a measure to quantify the relationship and proportion of alleles that comes from the reference species (In this case Spanish sparrow and house sparrow). The hybrid index ranges from 0 to 1, where pure house sparrow genotypes yield an index of 0 and pure Spanish sparrow genotypes 1. The hybrid index was retrieved by isolating DNA with Quiagen DNeasy 96 Blood and Tissue Kits (Quiagen N.V. Venlo, Netherlands) that were used according to the manufacturer's instruction. Three house sparrows and three Spanish sparrows of both sexes, representing the parental taxa, were sent to transcriptome sequencing. A cDNA library was constructed, and the genes were mapped to the zebra finch genome. Species informative SNPs, that is nucleotide sites where the Spanish sparrows and house sparrows appeared to be fixed for

different alleles according to the transcriptome sequence data, were chosen for genotyping. All individuals were genotyped using the Sequenom Mass ARRAY system at CIGENE, University of Life Sciences, Ås, Norway. This resulted in 81 species informative SNPs, from coding genes (see Trier et al. 2014 for further details), successfully genotyped on all individuals used in this study. The genotypes were used to estimate molecular hybrid index with the program Introgress 1.2.3 package in R (Gompert & Buerkle, 2012). The individuals classified as Italian sparrow in this study has a hybrid index ranging from 0.12 to 0.26 (Table 1), and the sparrows classified as house, have a hybrid index ranging from 0 to 0.06.

2.7 GLM

SPSS software (IBM Corp., 2012) was used for conducting GLM (general linear models). As a preliminary check a multivariate analysis was performed with centroid size (beak size), CV1 (beak shape component 1) and CV2 (beak shape component 2) as dependent variables. As response variables I used mean annual temperature, temperature seasonality, mean annual precipitation, precipitation seasonality and altitude. Multivariate and univariate GLMs with random factor and covariates were made to see what factors associates with the morphology of the beak. In the first univariate model for beak shape, I used as covariates, hybrid index, tarsus length and also all possible interactions of the environmental variables were tested. After performing a backwards selection, the best model for beak shape component 1 was: CV1 ~hybrid index + tarsus length. Beak shape component 2 and beak size were also used in univariate GLMs as dependent variables with the same variables, random factor and interactions. After backwards selection the best model for beak shape component 2 was: CV2 ~mean annual precipitation + mean annual temperature + tarsus length + temperature

seasonality. For beak size the best model was as dependent variables: centroid size ~ tarsus length + temperature seasonality + hybrid index + precipitation seasonality + mean annual precipitation.

2.8 Cline Analysis

Finally, I used geographical cline analysis to investigate if there are some emerging patterns between the shape and size of the beak, environmental variables, and the molecular hybrid index. A geographical cline analysis was chosen to infer if there is selection against hybrid beak morphology in the Alp hybrid zone due to gene flow or environmental factors. Each location's distance from the alpine ridge was used as the geographical distance. This was measured in Google Earth (Google Inc., 2013) following the procedure of Tesaker (2014) for those locations not included in Tesakers study. As parental populations, the most northern and southern populations were chosen. House sparrow parent locations were Kiesen, Thusis, Surrein, Ilanz and Andeer (N=17). Juf were excluded as this population is located at a very high altitude and thus makes a bias in the analysis of the environmental data in temperature and precipitation. Italian sparrow parent locations were Coldrerio, Uppone, Sciranna, Melide, Brusimpiano and Castel Veccana (N=22). The cline analysis was done on three response variables: beak size and both components of beak shape (CV1 and CV2), and mean values for these variables for the parental populations were calculated.

The cline analysis was performed in R (R Core Team, 2013) and Excel (Microsoft 2010) using a script developed by Dr. Richard Ian Bailey (see Tesaker 2014). In order to make the mean values for the house sparrows higher than the Italian sparrows, I transformed the values

for beak shape. This is necessary because they represent each side of the cline for the parent populations. Additionally, variance must be greater than 1. The cline analysis uses a logistic regression model and a maximum likelihood method based on a tanh curve function (1) to find the cline parameters; centre, width and standard deviation (i.e. dispersion parameter).

$$(1) \quad p = \frac{1}{2} [1 + \tanh (\frac{2[x-c]}{w})]$$

In the equation above (1), p = trait variable, x = distance from the Alpine ridge, c = cline centre, which is the point with highest slope value. w =width of the cline, which shows geographically where the change in the trait occurs. The values from the cline analysis were standardized and the means centered ($\mu=0$, $\sigma=1$), to make the visualization of the cline figures more comparable with each other. All cline figures were plotted by using the car package in R (Fox & Weisberg, 2011). I also used the cline analysis to investigate if I could find the centre and width of the environmental variables; temperature seasonality, mean annual temperature, precipitation seasonality, mean annual precipitation and altitude.

2.8.1 Strength of selection

The cline parameter width was further used to estimate the strength of selection against hybrids (Barton & Gale, 1993), and to calculate an estimate of life time dispersal. Width of the cline should scale to dispersal distance, as cline gets wider when dispersal increase and stronger selection leads to a narrower cline. I set average dispersal distance to 2 km, based on a review of Anderson (2006). By rearranging formula (2) I could estimate selection from formula (3). Additionally, I calculated selection estimates with dispersal distances of 5km and 10km.

$$(2) \quad w = 2 \frac{\sigma}{\sqrt{s}}$$

$$(3) \quad s = \left(\frac{2\sigma}{w}\right)^2$$

I also wanted to compare the widths from the cline analysis with artificial widths from the neutral diffusion model as derived by Barton & Gale (1993) (4). If clines occur due to secondary contact with neutral introgression the widths will be broader over time (Hewitt, 1988). If the widths based on equation (4), is much wider than those given from the cline analysis, it is likely to infer selection as one factor maintaining the hybrid zone in this area.

$$(4) \quad w = 2.51\sigma\sqrt{T}$$

$$(5) \quad T = \left(\frac{w}{2.51\sigma}\right)^2$$

In equations (4) and (5), T = number of generations since secondary contact, and σ = average life time dispersal. Hermansen et al. (2011) suggested the contact between the house sparrow and Italian sparrow to have happened at least one thousand generations ago. In addition to the cline analysis, environmental variables, namely, temperature seasonality, mean annual temperature, precipitation seasonality and altitude were also plotted along ridge distance to see if there is concordance between the cline parameters of beak size, beak shape and environmental variables.

3 Results

3.1 Beak shape and size differences

I found significant morphological differences in beak shape between the three categories house sparrow, Italian sparrow and hybrids. To summarize; the house sparrow has the narrowest lower mandible, and a straight, pointed and robust upper mandible. The Italian sparrow has a more curved upper mandible and a robust and broad lower mandible. Hybrids resemble the Italian in shape, but have a less curved upper mandible and a slightly reduced height of the lower mandible.

The CVA revealed significant differences in shape between each category. Beak shape component 1 (CV1) explains almost 65% of the total shape variation among the groups (Table 4), and beak shape component 2 (CV2) explains 35% of the variation in shape. Increasing value of beak shape component 1 implies a smaller lower mandible and a higher upper mandible, and also a more pointed beak (Fig. 3). Increasing values of beak shape component 2 implies a more robust and broader lower mandible, and a narrower upper beak, and also a more curved upper mandible (Fig. 3). The Italian sparrow has lower values of beak shape component 2 than the house sparrow and thus has a higher lower mandible compared to the house sparrow. The upper mandible is characterized by a change in shape from curved for low values of beak shape component 1 to straighter profile for higher values.

House sparrows have more slender lower mandible compared to both Italian sparrows and hybrids evident both from the CV scores and also the pairwise comparison from the

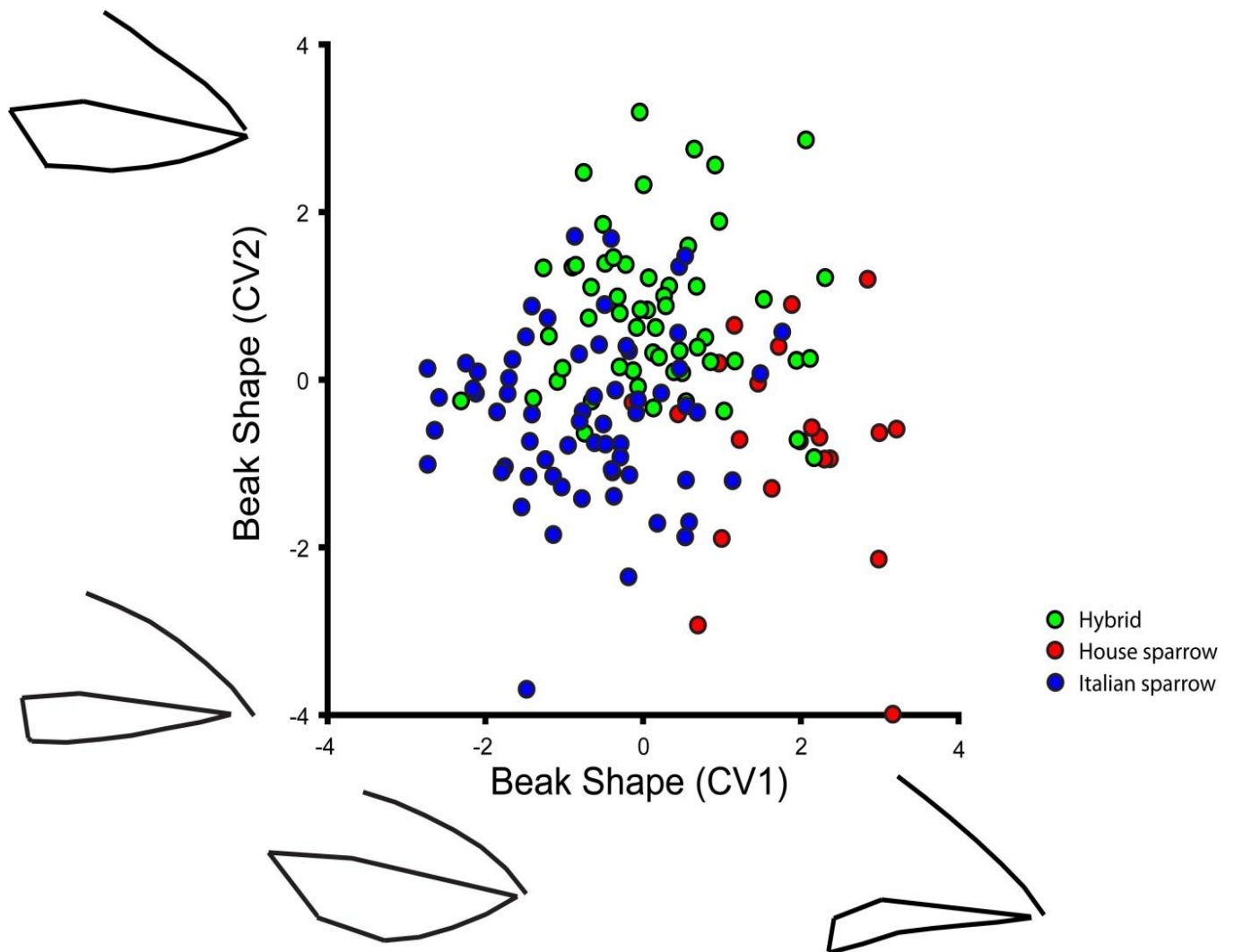


Figure 3. CVA scatter plot of the shape based on the canonical variate scores. Beak shape component 1 is on the x-axis and beak shape component 2 is shown on the y-axis. Hybrids are intermediate for beak shape component 1 and transgressive for CV2. The green, red and blue dots show the mean for each of the populations, hybrids, house sparrow and Italian sparrow respectively. Also the shape reflected by the CV axis is shown to visualize how the beak changes for the different values of CV1 and CV2, and represent changes in one direction of the axis by a factor of 10 to enable better visualization.

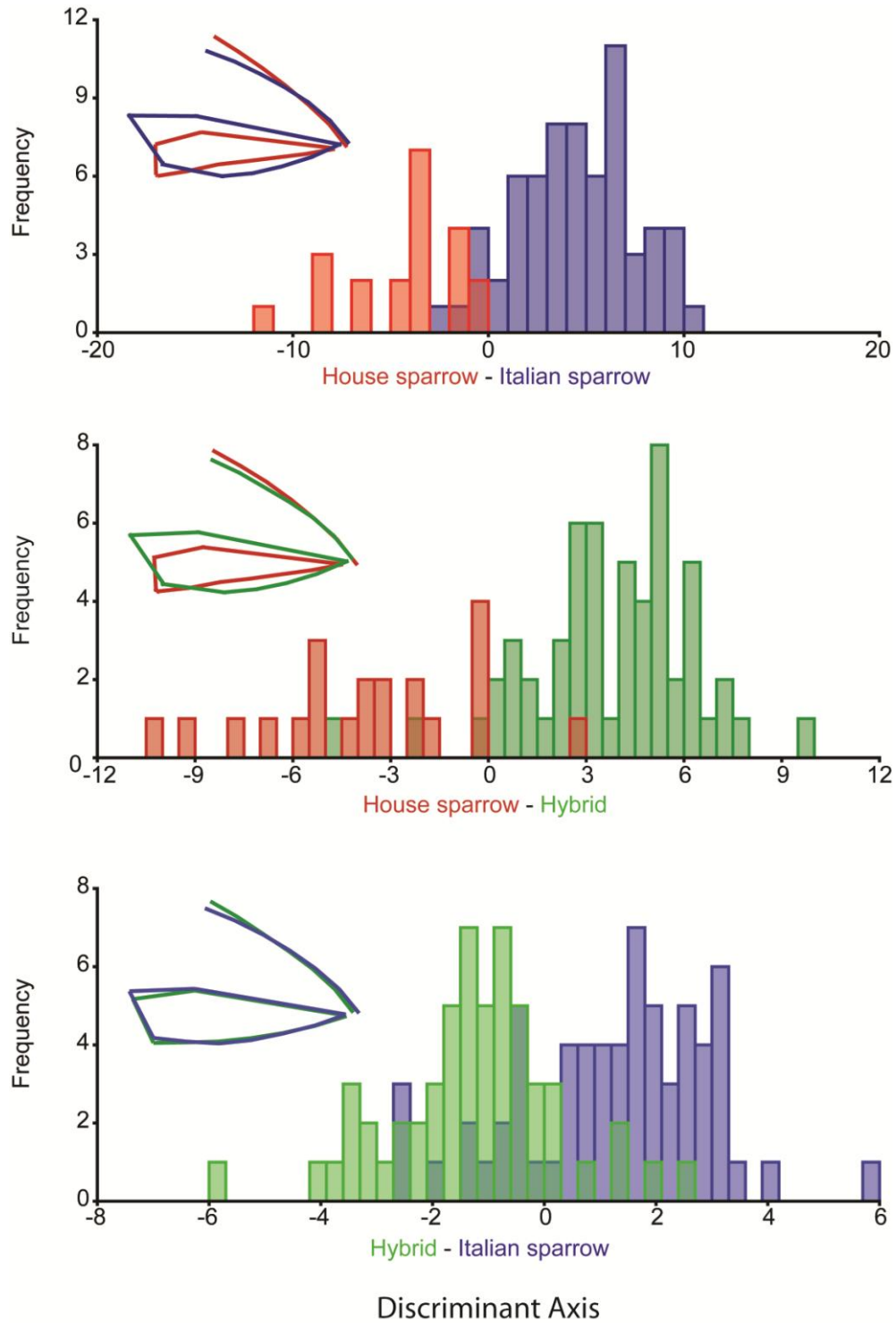


Figure 4. Pairwise comparison of the groups based on DFA. The three categories of individuals are separated from each other with outline of the beak shape to visualize the differences. Red is house sparrow, blue for Italian sparrow, and green is the hybrid. Top panel shows the comparison between house sparrow and the Italian sparrow. In the middle is house sparrow compared with the hybrid. Bottom panel is comparison between the hybrid and Italian sparrow.

discriminant function (see Fig 4). House sparrow has a straight upper mandible and this gives the beak a more pointed and robust profile, as evident from beak shape component 1 and also the discriminant function (Fig 4). Hybrids and Italian sparrow are more similar in shape with shortest Mahalanobis distance (Table 5), also shown in figures 4 and 5. Pairwise comparison between Italian sparrow and the hybrid reveals that the hybrids has a little more slender lower mandible and a more curved upper mandible, as evident from beak shape component 1 where the Italian has the lowest values (Fig. 3). Beak shape component 2 revealed another aspect of shape variation. Here the hybrids are discriminated from the parental species by having values exceeding the values of either of them (Fig. 3). Hybrids clearly show a pattern of transgression in this dimension. Shape change in relation to increasing values of beak shape component 2 is basically a more robust lower mandible and a more slender upper mandible.

Discriminant function analysis was performed in pairwise comparisons between the group means. Pairwise comparison between house sparrow and hybrids demonstrates that the species were well separated (Mahalanobis distance 2.75 ($p=0.0220$)). As much as 95.25% house sparrows were classified into the correct group, and 94.6% of the hybrids were correctly classified in the cross validation (Table 8). In the comparison between the house sparrow and the Italian sparrow (Table 7) the separation of the species was very good (Mahalanobis distance of 2.9830 and $p=0.001$). In the DFA cross validation, all house sparrows were correctly classified as morphological house sparrows, and 90.8% of the Italian sparrows were correctly classified as morphological Italian sparrows (Table 9). Differences between hybrids and Italian (Table 7) were not as clear as between the other two groups (Mahalanobis distance of 1.5914 and $p=0.0320$). In the DFA cross validation, 85.7% hybrids were classified as morphological hybrids and for Italian 76.9% were classified as morphological Italian sparrow (Table 10).

Beak size differences between the three categories are shown in figure 5. The house sparrow has the smallest beak size, hybrids are intermediate between the parents, and the Italian sparrow has the largest beak.

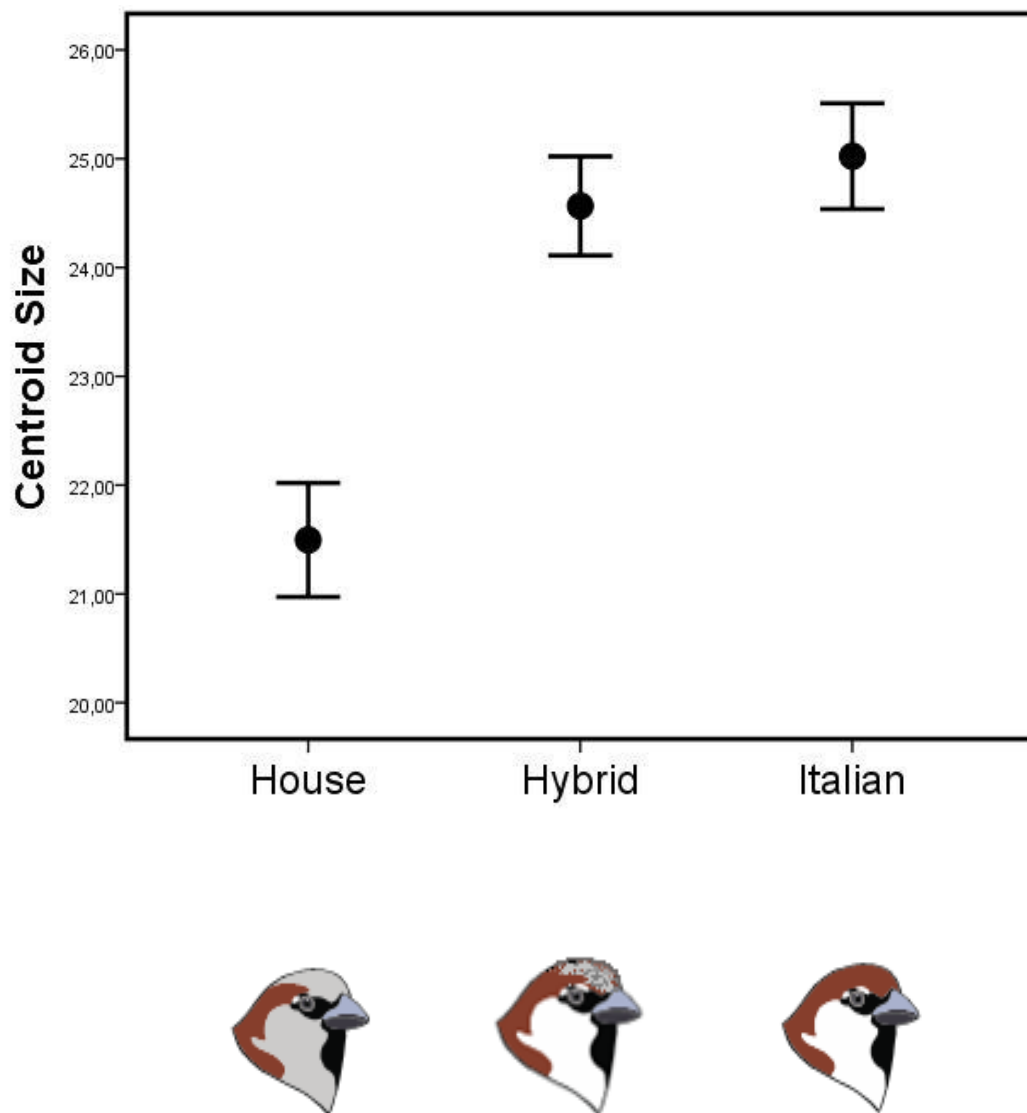


Figure 5. Beak size differences between the categories based on beak size. Hybrids are intermediate in beak size between the parent species, although it is closer in size with Italian which has the largest beak. House sparrow clearly has a much smaller beak.

3.2 Variation across the hybrid zone

MANCOVA analysis with beak size, and both components of beak shape as response variables, show that hybrid index, mean annual precipitation, precipitation seasonality, temperature seasonality and tarsus length to have a significant global effect on beak morphology (Tables 11 and 12). However, conducting univariate analysis (ANCOVA) on these three beak traits, revealed interesting patterns. Beak shape component 1 (intermediate) was significantly associated with hybrid index ($p < 0.001$) (Table 13), whereas beak shape component 2 (transgressive) was associated with mean annual temperature ($p = 0.031$), temperature seasonality ($p = 0.004$) and tarsus length ($p = 0.029$) (Table 14). Finally, beak size was affected by both allometric factors (tarsus length, $p = 0.027$), and ecological factors (temperature seasonality, $p = 0.006$; precipitation seasonality, $p = 0.005$, and mean annual precipitation, $p = 0.011$), and genetic factors (hybrid index, $p = 0.002$) (Table 15).

3.3 Cline Analysis

Results from the cline analysis are given in table 17. For the phenotypic traits beak size clearly has the sharpest cline with a centre at -20.21 and a width of only 6.86 km (Fig. 6, table 16). This centre is located between Malvaglia and Roveredo in southern Switzerland. The first component of beak shape also shows a cline, although not as steep as beak size, with a centre at -21.96 and a width of 18.78 km (Fig. 6, table 16). For the environmental variables altitude and annual temperature were concordant with both cline centers -17.32 and -17.59 and widths 5.53 and 4.06 respectively (Appendix 2, table 16). We could not perform cline

analysis for beak component 2 and mean annual precipitation, because they did not fit the model assumptions. Hybrid index has its centre at -15.28 and a width of 68 km (Table 16).

The selection estimates gives the proportion of drop in fitness of intermediate hybrid phenotypes compared to their parental species. Beak shape component 1 has a drop in fitness of 4.5 % compared to its parent species (Table 17), and for beak size the drop in fitness is 26% relative to its parent species (Table 17), which is 6 times more. Generation time estimates are all over very low. For the first beak shape component, generation time was 13.99, 2.24 and 0.56 for 2, 5 and 10 km dispersal (Table 17). For beak size the generation time estimates were 2.43, 0.39 and 0.10 for 2, 5 and 10 km dispersal (Table 17). With the neutral diffusion model, the estimation of what the width would be if the populations met 1000 generations ago is approximately 5000km.

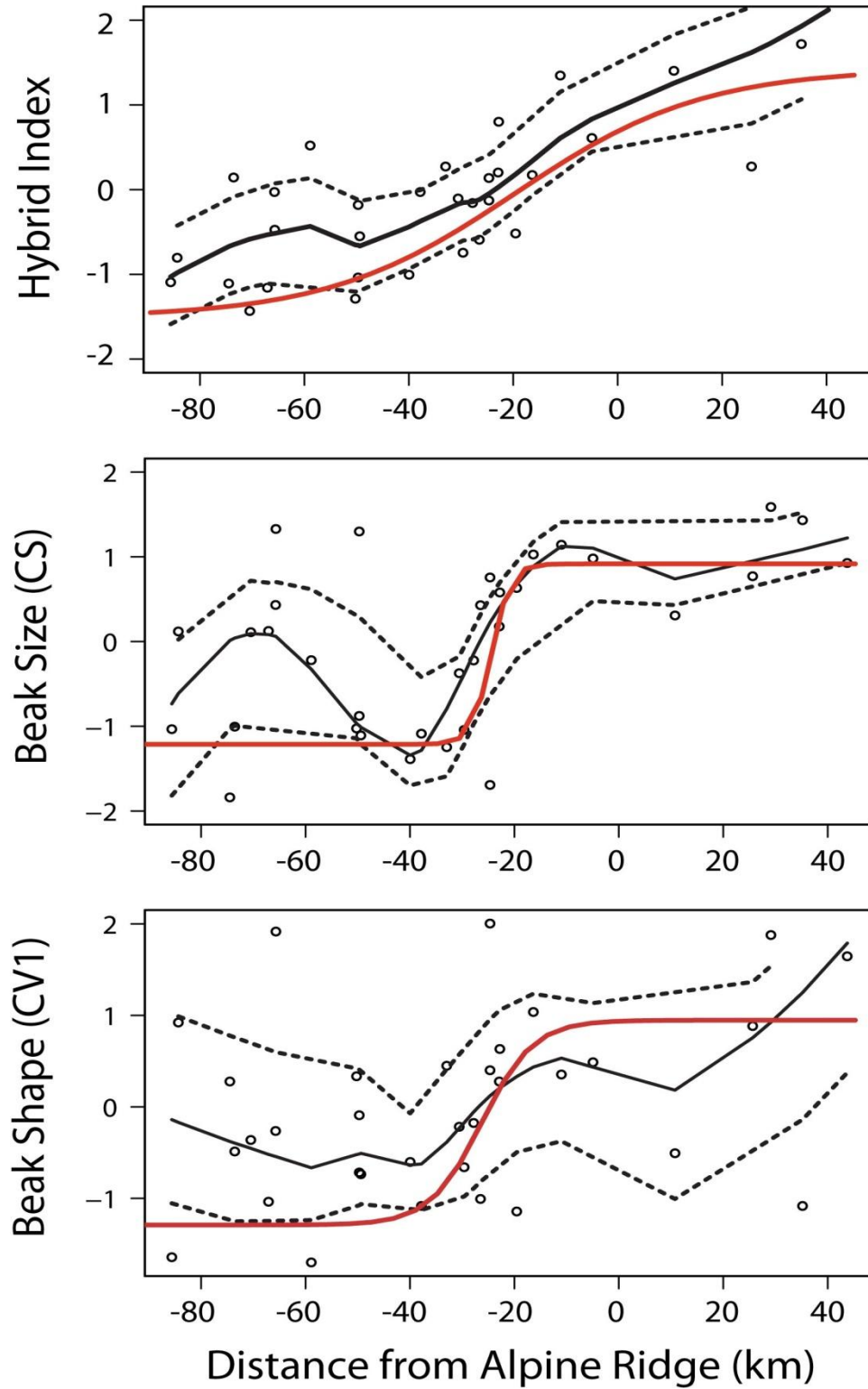


Figure 6. Cline for beak size (top panel) and shape (down) along the alpine transect. Distance from the Alpine ridge is shown on the x-axis, and the transformed values for the traits are shown on the y-axis. The red line is the predicted cline, black line is the estimated values from the analysis for the trait and the dotted lines are confidence intervals.

4 Discussion

The first aim of this study was to investigate whether there are differences in beak shape and size between the two sparrow species; the Italian sparrow, which is a hybrid species and the house sparrow, which is one of its parent species. It is interesting to see whether these two bird species, which share the same overall ecological niche, differ in any way in beak morphology. In addition, since these two species form a hybrid zone in the Alps, I also addressed whether current hybridization has an effect on beak morphology. Furthermore, I investigated whether environmental variation across the hybrid zone influences beak variation. Finally, I discuss whether beak variation may play a role in reproductive isolation.

4.1 Differences in Beak Morphology Between House and Italian Sparrow

Since the Italian sparrow and the house sparrow are seed eating, human commensalists one may predict them to be similar in beak shape and size. However, I found that they have significantly different beak shape and size. The house sparrows have a narrower lower beak and a more robust and pointed upper beak compared to Italian sparrows (Fig. 3). The Italian sparrow has a broad lower mandible and a curved upper beak. Most of the variation in shape for all samples strongly differentiates these two species. These two species also differ significantly in beak size, the Italian sparrow having a larger beak than the house sparrow.

Hybrid speciation can often lead to major changes in key ecological traits (Jiggins et al., 1996). Given that the same overall niche is shared by both species; it is possible that the differences I report here may not be a result to different adaptations. Differences in both size and shape may instead reflect the genetic contribution of Spanish sparrow (the other parent species) alleles in the Italian sparrow. This other parent species is less closely associated with humans compared to the house sparrow. The Spanish sparrow also strongly differs in beak size both to the house sparrow (Gonzales Rojas, 2012) and Italian sparrow (Eroukhmanoff et al., 2014). This difference in ecology is probably also responsible for displacement in beak integration when the two species occur in sympatry (Eroukhmanoff et al., 2014). Hence, it is interesting to notice these differences between house and Italian sparrows, and more work needs to be done in order to unravel the exact role of genetic parental contributions in beak morphology in this hybrid species.

4.2 The Effects of Hybridization on Beak Morphology

Past hybridization events may have had an effect on beak morphology, but in this study, I also had the opportunity to investigate the current effects of hybridization on beak morphology, since sampling was done in the heart of a hybrid zone between the focal species. Resulting hybrids, which unlike Italian sparrows, are early generation hybrids, were significantly different overall in beak shape from both species. They were intermediate in size and one component of shape, like the classic expectations for hybrid phenotypes (Grant & Grant, 1994), but with slightly more overlap with the Italian sparrow. However, by taking a closer

look, an interesting pattern emerged: with a combination of intermediacy in one dimension of shape and transgression in another one. More precisely, hybrids have a more straight upper beak than the Italian sparrow, but not as pointed as the house sparrow. Hence, they are intermediate between the parents in the values for beak shape component 1. Hybrids also have broader and more robust lower beaks compared to both their parents, which is reflected by the high scores in beak shape component 2. Transgressive segregation is when hybrids have a phenotype exceeding both parent species, and it is not an uncommon phenomenon (Seehausen, 2004). This can lead to the emergence of new phenotypes, which may be detrimental or advantageous in terms of fitness in different situations (Rieseberg et al., 1999). Transgression can be associated with hybrid-superiority, heterosis and colonization of new habitats, although this is quite rare (Rieseberg, 2000,). Contrary, transgression can also have negative effects, like extreme maladapted phenotypes with lower fitness, which can contribute to isolation and selection against hybrids (Grant & Grant, 1994).

This combined pattern of both phenotypic intermediacy and transgression for different shape characters has also been shown in other studies, like for the mouse mandible (Renaud et al., 2009), where the condyle is intermediate and both the angular and molar zone are transgressive. This may be due to many genes involved. If it is additive genetic effects the hybrid is expected to be intermediate between the parent species (Klingenberg et al., 2001; Renaud et al., 2012). Non-additive effects can generate new morphological phenotypes that are transgressive (Rieseberg et al., 1999). The complex pattern of intermediacy and transgressive traits can also indicate modularity (Klingenberg et al., 2004) where different parts of the trait is controlled by different genes that develop independently of each other.

The similar pattern of intermediacy and transgression found in mice and sparrows suggests that the phenotypic effects of hybridization between a hybrid species and its parent are

comparable to more classic cases of hybridization, at least when it comes to beak morphology. The pattern of intermediacy and transgression was confirmed by evaluating the links between individual levels of genetic admixture and shape components, which revealed that the first shape dimension was correlated with hybrid index, hence signalling that phenotypic intermediacy is reflected at the genetic level. However, the transgressive component of shape did not correlate with hybrid index, suggesting that variation along this shape dimension may either be due to the interaction of a few genes localized in a specific region affecting beak morphology (and therefore not being captured by genome wide levels of admixture), or by extrinsic factors such as environmental variation.

4.3 The effects of environmental variation on beak morphology

Beak shape and size may covary strongly with climate variation (Grant & Grant, 2002; Hendry, 2009; Eroukhmanoff et al., 2013). In such a sharp environmental transition as the Alps, it was thus logical to investigate whether beak morphology was in any way correlated with variation in temperature or precipitation variables. I found that beak size is strongly associated with precipitation regimes, like it has been shown before for the Italian sparrow in other parts of Italy (Eroukhmanoff et al., 2013). However, temperature seasonality also has an effect on beak size, probably due to the fact that there is more variation in this factor in the Alps than in the rest of Italy (Eroukhmanoff et al., 2013). Furthermore, temperature also had a strong effect on the transgressive dimension of beak shape. For the other dimension of shape none of the environmental factors had any effect. Beak size and shape is greatly influenced by

foraging. The continuous gradient in both temperature and precipitation over the Alps may affect the availability of what kind of seed or seed size they feed on. Moreover, the major variation in landscape in this hybrid zone, from open flat fields with large areas of cultivated land, to narrow valleys up to the alpine ridge, where animal farms and grazing land is dominating, most likely affects the food resources for the sparrows. Thus, this may affect beak morphology in various ways if populations can evolve towards their fitness optima. So it is not so surprising to find such correlations between climate and beak size and shape. It may be hybrid-superiority (Moore, 1977) that maintains this hybrid zone, because hybrids may be more fit, if only in a narrow geographic zone, than their parents.

4.4 A potential role of beak morphology in reproductive isolation

The cline analysis revealed a steep cline for beak size, and cline width was only 7 km, indicating that the shift between the two parent species in beak size happens over a small area. This is almost 5 times narrower than cline width found for a plumage trait that has been interpreted to play a role in reproductive isolation between these same two species in this same hybrid zone, namely crown colouration (Tesaker, 2014). This suggests that beak size may also contribute to a strong reduction in gene flow between the species. It is interesting to notice that beak size have also been shown to be involved in mate choice in the house sparrow (Kimball, 1996) and in isolation by adaptation in the Italian sparrow (Eroukhmanoff et al., 2013). In comparison, I also found a cline for beak shape, although the cline width was slightly larger than for size. When estimating selection coefficients the results shows larger drop in fitness for beak size compared to shape, indicating stronger selection on beak size

than shape. One possibility is that one dimension of beak size, e.g. beak height may contribute to gene flow (Eroukhmanoff et al., 2013). In comparison the cline for *B. Bombina* and *B. Variegata* is similarly narrow, only 6km (Szymura et al. 1986), which also have a cline which indicates a barrier to gene flow within the hybrid zone.

Additionally the generation time estimates indicates that this would have to be a very new hybrid zone if the traits measured are not under selection (only 13 generations for shape). Hybrids between Italian sparrow and house sparrow have been seen at more than 100 years ago in this area (Wallis, 1888). Low generation time estimates thus indicate strong selection. Earlier studies have suggested that this hybrid zone has its origin some thousand generations ago (Hermansen et al., 2011).

Generation time estimates assuming neutral diffusion are much lower than the likely age of the hybrid zone for the clines of beak size, beak shape and also for crown, so it is not likely that these clines are maintained by neutral diffusion following secondary contact. This is because according to the neutral diffusion theory with no selection, clines will flatten out over time, yielding very short time estimates for my steep clines (Barton & Gale, 1993; Endler, 1977).

For the traits and environmental factors in this study, and also for the plumage in Tesaker (2014) there is some variation in cline width. Although size, altitude and annual temperature have narrow widths (4-7 km), crown (Tesaker, 2014) and precipitation seasonality have concordant widths (32-36 km), and also hybrid index and temperature seasonality have the same widths (58-68 km). Different ecology for the hybrids compared to their parental species can result in ecological selection due to different types of agriculture or food the beak would adapt to. Hence hybrids can be more fit within this geographical transition of climatic factors, giving selection against the parental species within this zone. This is referred to as the hybrid-

superiority hypothesis (Moore, 1977). In parts of the hybrid zone the hybrids may be more fit than their parentals, and it will be selection against the parental phenotypes. According to this hypothesis the range of the hybrid zone is in concordance to the width of the ecotone (Moore & Price, 1993). Furthermore, if the geographic range for the distribution of the parental phenotypes are located to the hybrid zone, for example if the northern border for the Italian phenotype is determined by temperature, it will not move further north or cross the Alps. Alternatively, interspecific competition may prevent the house sparrow from expanding further into the Italian peninsula. If the hybrid zone is located at the geographic range of environments the Italian sparrow can cope with, low density can also be a factor leading to hybrids in this contact zone, because lower population density can hamper mate choice, and thus lead to hybridization. Under this scenario selection against hybrids will be strong outside this contact zone, giving a barrier to gene flow, north and south of the contact zone. Possibly the high age of this hybrid zone can be due to migration-selection equilibrium (Barton & Hewitt, 1985). Thus, the influx of parental alleles and selection against hybrids are kept in balance causing stability, a pattern also referred to as the tension-zone model (Haldane, 1948). Recombinant genotypes can lead to a pattern of clinal variation (Teeter et al., 2008). There are strong differences in cline widths, for beak size and beak shape compared to a plumage trait under selection: crown (Tesaker, 2014) or to a genome wide admixture index: hybrid index. This may be due to different levels of introgression at different traits in this hybrid zone, caused by different strengths of selection against intermediates (see also Brumfield et al., 2001). Beak size, which has the narrowest width appears to under stronger selection than beak shape and crown, and may be creating a barrier that prevents gene flow.

Because the cline centres for beak size, beak shape, hybrid index and crown is located at approximately the same location south of the alpine ridge, between Malvaglia and Roveredo, this is most likely the centre of this hybrid zone. Coincident cline positions, like what is found

for beak size, beak shape, hybrid index and crown in this system, can on the other hand be an indication of genome wide barriers to gene flow (Ruegg, 2008). In summary, in this system there is evidence for coincident position of cline centres for several traits, but they differ in widths. This suggests several traits are selected against in hybrids, but at different strengths.

However, these strong results may also reveal another process at hand. Indeed, environmental variation could itself have generated such a pattern, and the steep clines observed may only reveal strong local adaptation. Interestingly, the cline centres of both beak size and shape are situated between the cline centres for altitude and mean annual temperature and the cline centres for precipitation and temperature seasonality. The centres for beak size and shape, which are situated between Malvaglia and Roveredo, also occur along a shift in landscape, where the narrow valleys start leading up to the alpine ridge. Thus, there is reason to believe that these clines are maintained in this hybrid zone by environmental factors. Nevertheless, if the pattern I found is mostly due to populations tracking their fitness optima along this hybrid zone, this will still result in an efficient barrier to gene flow, as migration from parent populations will be strongly reduced due to mismatched beak phenotypes.

5 Conclusion

In this study I investigated differences in beak morphology between the Italian sparrow and house sparrow, in a contact zone in the Alps. I used geometric morphometrics to investigate differences between them. An interesting pattern that hybrids between these two species are intermediate in beak size and one dimension of beak shape was found. Furthermore, both environmental factors in various ways, and also genetic background were found to have affected these two traits. Additionally, steep clines, and low generation time estimates indicates strong selection on these intermediate traits. Contrary, transgression was found in another dimension of beak shape, only temperature and precipitation seems to be important for this trait. This suggests that genetic background and environment are involved in different modules in the development of the beak.

I found that beak size and shape may be involved in reproductive isolation. It would be interesting to investigate further into beak morphology in the sparrows, whether it is as important in hybridization and eventually speciation as in Darwin's finches (Grant & Grant, 1994; Hendry, 2009). Future perspectives would be to link the genetics, by using a candidate gene approach, to the development of these morphological traits. Also 3-D data in addition to incorporate the whole skull in future studies will give a more complete insight of this morphology and modularity of this important ecological trait. This can be useful in order to understand how covariation between different parts of the beak during development may affect evolvability and a potential change in morphology. Ultimately, this will allow to better investigate how environment and genetic background contribute to the evolution of this trait.

6 Glossary

Bending energy	A measure of how much energy is used in non-uniform shape change. It takes more energy to change shape in landmarks that are close, than landmarks that are more far away from each other.
Centroid size	A standard measure of size in geometric morphometrics which is defined by the sum of distances from the centre to each landmark. Calculated from original landmark coordinates.
Consensus	Mean shape of all the samples, showing the distribution of all the landmarks in relation to a mean sample.
Landmark	Specific point on an image, which has a well-defined location consistent between all specimens (homologous).
MorphoJ	Program for statistical analysis of geometric morphometric data acquired through tpsDig2 or similar programs.
Outline	Curve defining a physical boundary of a specimen.
Partial warp	Linear combinations of the principal warps describing the particular shape change in landmark position for one coordinate.
Principal warp	Starting point of the calculations used to characterize any shape change.
Procrustes distance	The sum of distances between the corresponding landmarks of the shapes.
Procrustes superimposition (GPA)	Rescales, translates and rotates every specimen's landmark configuration to remove effects of size, and make the configurations comparable to each other by putting them in the same coordinate system. This promotes the investigation of the shapes, because distances are minimized to ensure that differences found are true differences in shape.
Relative warp	Principal components of the partial warps.
Semi-landmark	Landmarks which are defined by homologous landmarks, or used as points to get information about an outline, so they are not as reliable as homologous landmarks because they have fewer degrees of freedom.
tpsDig2	Program used for data acquisition, digitizing landmarks with features to enhance images and scaling.
tpsRelw	Program used to make consensus configuration, relative warps and partial warps to be used in further analysis.
tpsUtil	Utility program used to create an empty tps file in the same directory as the images. Also for re-ordering, deleting specimens and adding/removing links, sliders or semi landmarks.

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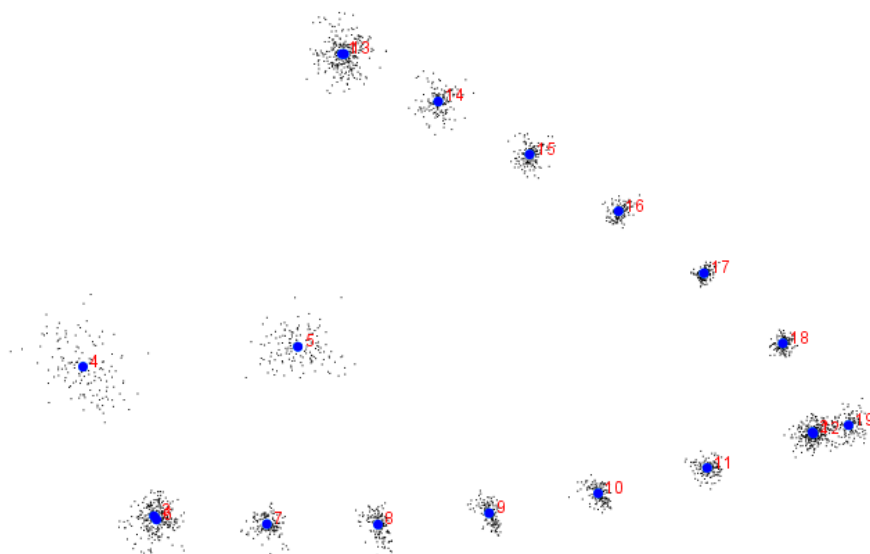
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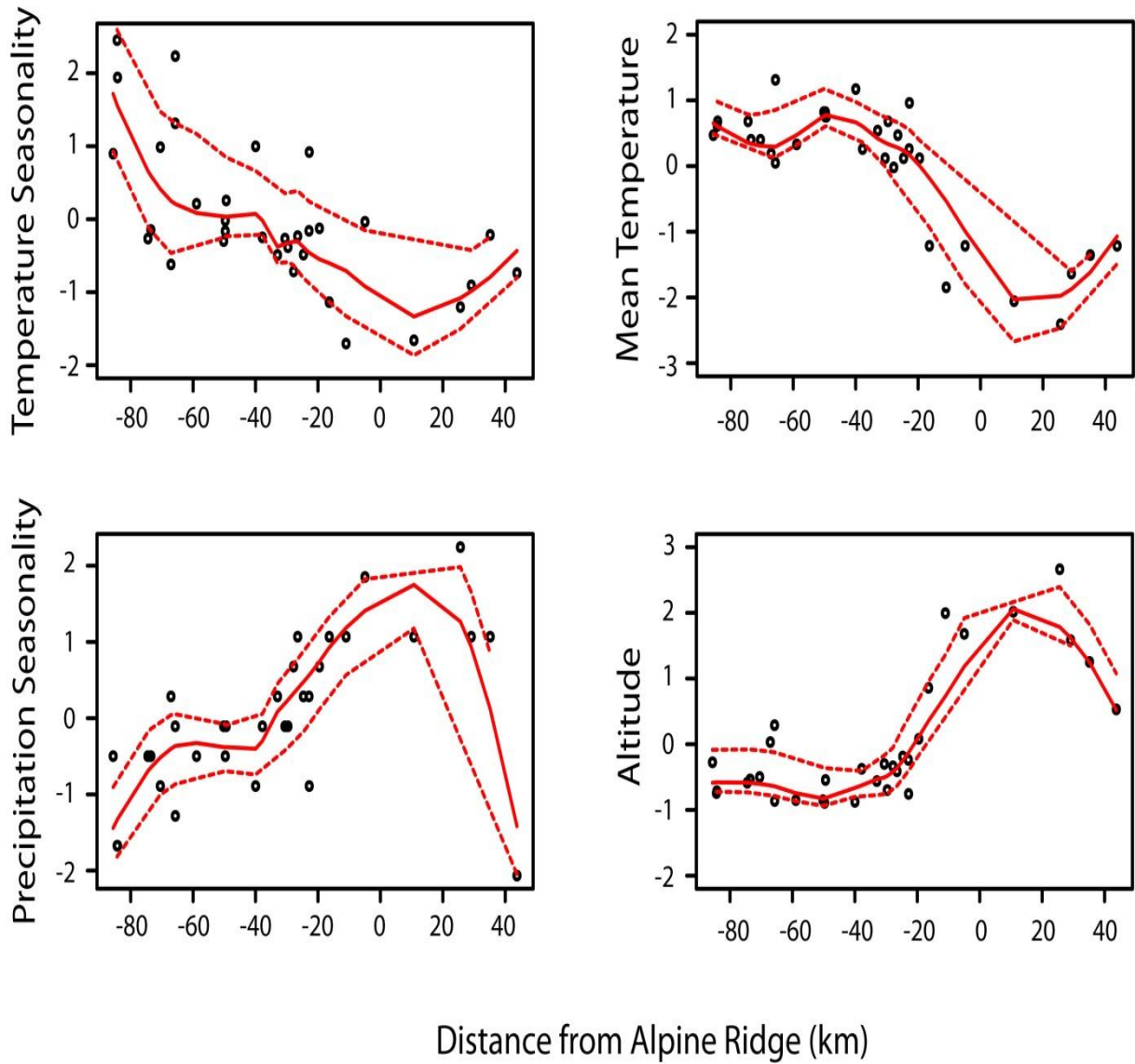
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8 Appendices



Appendix 1. Consensus plot showing the variation of all specimens landmarks on the beak (the black dots). The blue dot is the mean configuration of the landmark.



Appendix 2. Graphs showing the climatic variation along the Alpine transect. The x-axis is the distance to the alpine ridge, and the y-axis is the transformed values of the environmental variables. Upper left is temperature seasonality, upper right is the mean annual temperature. Bottom left is precipitation seasonality and bottom right is the altitude.

9 Tables

Site	Species	Latitude	Longitude	Altitude	Sample size	Ridge distance (km)	Hybrid index. pop mean.
Andeer	<i>P. domesticus</i>	46.6141	9.4386	1055	1	25.32	0.011
Aurigeno	Hybrid	46.235	8.7125	323	11	-37.8*	0.123
Bellinzona South	<i>P. italiae</i>	46.174	8.9819	208	6	-50.21*	0.193
Bondo	Hybrid	46.3352	9.5531	818	1	-4.94*	0.108
Brusimpiano	<i>P. italiae</i>	45.9460	8.8901	284	5	-73.54*	0.247
Campagnola	Hybrid	46.2767	9.3898	231	4	-22.79*	0.216
Castel Veccana	<i>P. italiae</i>	45.9488	8.6777	293	5	-70.5*	0.167
Castro	Hybrid	46.4770	8.9327	620	2	-16.38*	0.155
Claro	Hybrid	46.2493	9.0172	245	8	-29.62*	0.209
Coldrerio	<i>P. italiae</i>	45.8523	8.9863	347	3	-85.61*	0.113
Gravedona	<i>P. italiae</i>	46.1303	9.2891	202	7	-39.97*	0.158
Ilanz	<i>P. domesticus</i>	46.7658	9.2093	735	1	29.12	0
Iragna	Hybrid	46.3271	8.9732	278	11	-32.96*	0.152
Juf	<i>P. domesticus</i>	46.4455	9.5790	2182	2	4.5*	0.008
Kiesen	<i>P. domesticus</i>	46.8038	7.5752	541	8	43.76*	0.021
Lecco South	<i>P. italiae</i>	45.7826	9.4237	217	4	-66.09*	0.256
Lirone	Hybrid	46.3676	9.3613	860	2	-14.98*	0.138
Locarno Camping	<i>P. italiae</i>	46.1554	8.8003	198	5	-45.13*	0.163
Locarno Piano	<i>P. italiae</i>	46.1690	8.8934	198	8	-49.69*	0.145
Lodano	Hybrid	46.2630	8.6858	341	1	-34.96	0.189
Lostallo North	Hybrid	46.3218	9.2008	433	1	-19.53*	0.227
Maccagno	<i>P. italiae</i>	46.0422	8.7366	208	2	-58.9	0.220
Malvaglia	Hybrid	46.4045	8.9792	368	2	-24.65*	0.153
Mandello Del Lario	<i>P. italiae</i>	45.9131	9.3156	205	2	-64.69*	0.112
Melide	<i>P. italiae</i>	45.9523	8.9499	272	2	-74.47*	0.150
Olivone	Hybrid	46.528	8.9341	894	1	-11*	0.086
Personico	Hybrid	46.3735	8.9114	332	5	-27.8*	0.168
PianiDi Verdabbio	Hybrid	46.2831	9.1738	356	1	-22.87*	0.176
Porlezza	<i>P. italiae</i>	46.0341	9.1348	282	3	-49.39*	0.201
Roveredo	Hybrid	46.241	9.1374	314	6	-26.49*	0.157
Savosa	<i>P. italiae</i>	46.0201	8.9419	421	3	-67.09*	0.122
Sciranna	<i>P. italiae</i>	45.8024	8.779	241	5	-84.21*	0.168

Surrein	<i>P. domesticus</i>	46.7215	8.9444	899	1	10.75	0.064
Thusis	<i>P. domesticus</i>	46.6960	9.4407	715	6	35.17*	0.055
Upponne	<i>P. italiae</i>	45.7905	8.5878	234	2	-84.34	0.199
Valbrona	<i>P. italiae</i>	45.8685	9.2859	483	1	-65.72*	0.169

Table 1. Table showing sampling locations, species, sample size, latitude, longitude, altitude, distance to the Alpine ridge and hybrid index (population mean) for each of the locations in the Alps hybrid zone. * shows ridge distances retrieved from Tesaker (2014).

Repeatability Statistics

Cronbach's Alpha	N of items
0.967	2

Table 2. Cronbach's Alpha coefficient in the repeated measures is very high, 0.967, and therefore the landmark data are considered to be precise and reliable.

	Intraclass Correlation	95% Confidence Interval		P
		Lower Bound	Upper Bound	
Single Measures	0.936	0.911	0.954	<0.001
Average Measures	0.967	0.954	0.976	<0.001

Table 3. Correlation coefficients for the repeatability test with confidence intervals and p-values.

CVA

CVA axis	Eigenvalues	% Variance	Cumulative
CV1	0.7665	64.961	64.961
CV2	0.4134	30.039	100

Table 4. Table showing the variation among groups based on the CVA analysis, with eigenvalues and % variance explained by each of the CV axis. CV1 explains approx. 65% of the variance and CV2 explain 35%.

Pair	Mahalanobis distance	P-values from permutation test
D-H	2.2405	<0.001
H-I	1.5128	<0.001
D-I	2.5758	<0.001

Table 5. Mahalanobis distances from the CVA, among the groups with p-values for the permutation test. House sparrow (D), Italian sparrow (I) and hybrids (H). Largest distance is between house and Italian sparrow, and Italian and hybrids have the shortest distance of the groups.

Discriminant Function Analysis

Difference between means						
	D-H	p-value for perm test	D-I	p-value for perm test	H-I	p-value for perm test
Mahalanobis distance	2.7509		2.9830		1.5914	
T-square	115.5754	0.0220	141.2377	0.0010	76.1846	0.0320
P-value (parametric)	0.0240		0.0014		0.0384	

Table 6. Discriminant functions analysis separated the groups with all over significant p-values as shown in the table, and Mahalanobis distance to be largest between the Italian and house sparrow.

Classification /misclassification table			
Group 1 : D			
Group 2 : H			
True	Allocated to		
Group	Group 1	Group 2	Total
Group 1	20	1	21
Group 2	3	53	56

Table 7. Classification table that shows 20 of 21 house sparrows (D) were assigned into correct group, and 53 of 56 hybrids (H), were correctly classified into correct group from the cross validation in DFA.

Classification /misclassification table			
Group 1 : D			
Group 2 : I			
True	Allocated to		
Group	Group 1	Group 2	Total
Group 1	21	0	21
Group 2	6	59	65

Table 8. Classification table that shows all house sparrows (D) were classified into the correct group in the DFA. For Italian sparrow (I) only 6 of 65 individuals were misclassified into the house sparrow group.

Classification /misclassification table			
Group 1 : H			
Group 2 : I			
True	Allocated to		
Group	Group 1	Group 2	Total
Group 1	48	8	56
Group 2	15	50	65

Table 9. Classification table showing 48 of the 56 hybrids (H) was classified into the correct group, and 50 of the 65 Italian sparrows (I) were correctly classified as Italian sparrow in the DFA.

MANCOVA

Variable		$F_{3,27}$	P
Hybrid Index	Pillai's Trace	16.34	<0.001
Tarsus Length	Pillai's Trace	4.43	0.012
Temperature Seasonality	Pillai's Trace	3.42	0.031
Mean Annual Precipitation	Pillai's Trace	5.01	0.007
Precipitation Seasonality	Pillai's Trace	4.81	0.008

Table 10. MANCOVA from the multivariate test with p-values for Pillai's trace. Multivariate test with beak size, beak shape component 1 and 2 as response variables, and hybrid index, tarsus length, temperature seasonality, mean annual precipitation and precipitation seasonality as explanatory variables. Weighted least squares regression is used to weight for sample size.

Variable	Dependent variable	SS	F_{1,29}	P
Hybrid index	CV1	38.09	41.85	<0.001
	CV2	2.01	0.99	0.327
	Beak size	71.62	11.81	0.002
Tarsus length	CV1	3.59	3.94	0.057
	CV2	13.28	6.57	0.016
	Beak size	32.78	5.40	0.027
Temperature Seasonality	CV1	1.22	1.34	0.256
	CV2	6.10	3.02	0.093
	Beak size	54.29	8.95	0.006
Mean Annual Precipitation	CV1	5.56	6.11	0.020
	CV2	3.05	1.51	0.229
	Beak size	45.40	7.48	0.011
Precipitation Seasonality	CV1	5.81	6.38	0.017
	CV2	0.14	0.07	0.791
	Beak size	55.63	9.17	0.005

Table 11. Multivariate test showing the response variables with sum of squares, f-statistics and p-values for each explanatory variable. Beak shape component 1 (CV1), beak shape component 2 (CV2) and beak size are response variables and several environmental variables are used as explanatory variables. Weighted least squared regression is used to weight for sample size.

ANCOVA

Beak shape component 1

Variable	SS	F _{1,32}	P
Hybrid index	75.66	66.71	<0.001
Tarsus length	3.11	2.74	0.108

Table 12. ANCOVA test for beak shape component 1, with sum of squares (SS), F-statistics and p-values. Weighted least squares regression used to correct for sample size. R Squared =0.717. This component of beak shape is significant for hybrid index, showing that genetic is important for this trait.

Beak shape component 2

Variable	SS	F _{1,30}	P
Mean Annual Precipitation	6.49	3.73	0.063
Mean Annual Temperature	8.86	5.10	0.031
Tarsus length	9.17	5.28	0.029
Temperature Seasonality	17.43	10.04	0.004

Table 13. ANCOVA performed on beak shape component 2. With sum of squares(SS), F-statistics and p-values. For beak shape component 2; temperature seasonality, tarsus length and mean annual temperature are significant with p-values < 0.05. R Squared = 0.355. This transgressive trait is influenced of environments, and not hybrid index.

Beak size

Variable	SS	F_{1,29}	P
Tarsus Length	32.779	5.403	0.027
Temperature Seasonality	54.288	8.948	0.006
Hybrid Index	71.618	11.805	0.002
Precipitation Seasonality	55.638	9.171	0.005
Mean Annual Precipitation	45.397	7.483	0.011

Table 14. ANCOVA for beak size showing sum of squares (SS), f-statistic and p-values. Weighted least squares regression is used to correct for sample size. For beak size tarsus length, temperature seasonality, hybrid index, precipitation seasonality and mean annual precipitation is significant with p-values <0.05. R Squared=0.618.

Cline Analysis

Variable	Centre	Width	SD
Hybrid index	-15.28	68.02	3.33
Beak shape (CV1)	-21.96	18.78	0.97
Beak size	-20.21	6.86	2.51
Precipitation seasonality	-34.89	32.68	1.97
Temperature seasonality	-36.01	57.88	132.34
Mean annual temperature	-17.59	4.06	0.51
Altitude	-17.32	5.53	98.94

Table 15. Cline parameters width, and centre with standard deviation for all traits and environmental variables used in the cline analysis.

Variable	Width	Centre	Dispersal	S	Gen. time
Beak shape	18.78	-21.96	2	0.045	13.99
			5	0.284	2.24
			10	1.134	0.56
Beak size	6.86	-20.21	2	0.261	2.43
			5	1.631	0.39
			10	6.524	0.10

Table 16. Cline parameters for break shape and beak size used from the cline analysis. S is selection coefficient based on dispersal distance, generation time estimate is based on the widths from the cline analysis and dispersal distance in km. Very low generation time estimates indicates selection for these two traits.

